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ORIGINAL PAPER

Cryopreservation of ectomycorrhizal fungi has minor effects on root colonization of *Pinus sylvestris* plantlets and their subsequent nutrient uptake capacity

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Abstract The use of ectomycorrhizal (ECM) fungi for afforestation, bioremediation, and timber production requires their maintenance over long periods under conditions that preserve their genetic, phenotypic, and physiological stability. Cryopreservation is nowadays considered as the most suitable method to maintain the phenotypic and genetic stability of a large number of filamentous fungi including the ECM fungi. Here, we compared the ability of eight ECM fungal isolates to colonize *Pinus sylvestris* roots and to transport inorganic phosphate (Pi) and NH_4^+ from the substrate to the plant after cryopreservation for 6 months at −130 °C or after storage at 4 °C. Overall, the mode of preservation had no significant effect on the colonization rates of *P. sylvestris*, the concentrations of ergosterol in the roots and substrate, and the uptake of Pi and NH_4^+. Comparing the isolates, differences were sometimes observed with one or the other method of preservation. *Suillus bovinus* exhibited a reduced ability to form mycorrhizas and to take up Pi following cryopreservation, while one *Suillus luteus* isolate exhibited a decreased ability to take up NH_4^+. Conversely, *Hebeloma crustuliniforme*, *Laccaria bicolor*, *Paxillus involutus*, and *Pisolithus tinctorius* exhibited a reduced ability to form mycorrhizas after storage at 4 °C, although this did not result in a reduced uptake of Pi and NH_4^+. Cryopreservation appeared as a reliable method to maintain important phenotypic characteristics (i.e., root colonization and nutrient acquisition) of most of the ECM fungal isolates studied. For 50 % of the ECM fungal isolates, the colonization rate was even higher with the cultures cryopreserved at −130 °C as compared to those stored at 4 °C.

Keywords Cryopreservation · Ectomycorrhizal fungi · Root colonization · Pi and NH_4^+ transport · 4 °C storage

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Introduction

Ectomycorrhizal (ECM) fungi are key organisms in forest ecosystems (Smith and Read 2008). Their application in timber production, afforestation, and bioremediation, and for some species, their production as edible mushrooms are increasing continuously (Fazenda et al. 2008; Duponnois et al. 2011; Karwa et al. 2011; Siddiqui and Kataoka 2011; Repáč 2011). However, the utilization of ECM fungi requires the selection of strains of high biotechnological and economical value and their subsequent preservation over long periods under conditions that maintain their genetic, phenotypic, and physiological characteristics (Kuek 1994; Ryan et al. 2001; Ryan and Smith 2004; Stacey and Day 2007; Duponnois et al.

2011; Repáč 2011). Any change with respect to their capacity to establish a functional symbiosis would indeed partly compromise their biotechnological value. Moreover, it can be hypothesized that an ECM fungus with impaired vital functions would be less competitive under field conditions and become more vulnerable to adverse environmental conditions.

Continuous subculture on agar slants is the most common method used to preserve ECM fungi in culture collections (Brundrett et al. 1996; Kumar and Styanarayana 2002; Repáč 2011; Siddiqui and Kataoka 2011). This method consists in preserving ECM fungal stock cultures on nutrient agar Petri dishes/slants, usually at 2–5 °C, and in transferring them at regular intervals onto fresh synthetic medium at 20–25 °C before storage at 2–5 °C. However, this method has been reported to induce a decrease in the ability of fungal isolates to form ECM associations and to reduce the effectiveness of the fungi to improve plant growth (Laiho 1970; Marx and Daniel 1976; Thomson et al. 1993). In this context, cryopreservation has often been suggested as a reliable alternative for long-term preservation. Preservation at ultralow temperature is considered as the most reliable method for the long-term storage of filamentous fungi (Smith 1998) as it does not stimulate mutagenesis compared to other preservation procedures (Hubálek 2003) and thus allow extended periods of storage (Mazur 1984). However, cryopreservation of ECM fungi is not widespread since many of them do not survive or exhibit poor recovery after freezing (Ito and Nakagiri 1996; Corbery and Le Tacon 1997; Smith 1998; Yang and Rossignol 1998; Danell and Flygh 2002). ECM fungi do not produce conidia (Hutchison 1989), and the sexual structures are rarely formed on synthetic media. Their most common form under in vitro culture conditions is vegetative mycelium that generally grows very slowly in culture and is very sensitive to environmental conditions (Heinonen-Tanski 1990; Molina et al. 2002; Homolka et al. 2003). In this context, a suitable and easy-to-apply cryopreservation protocol based on the direct growth of the fungal mycelium in cryovials was recently developed at the Mycothèque de l'Université Catholique de Louvain[1] for the preservation of a large set of ECM fungal isolates (Crahay et al. 2013).

Nowadays, studies reporting on the cryopreservation of ECM fungi are mostly focused on the evaluation of viability and, for some species, on their micro- and macro-morphological characteristics (e.g., growth rates, area of the colonies,…) following storage at ultralow temperature (Ito and Nakagiri 1996; Corbery and Le Tacon 1997; Yang and Rossignol 1998; Danell and Flygh 2002; Obase et al. 2011; Stielow et al. 2012; Crahay et al. 2013). To our knowledge, only one study reported on the ability of ECM fungi to re-establish a functional symbiosis after cryopreservation, while none reported on the capacity/efficiency of cryopreserved ECM fungi to sustain host plant nutrition. Corbery and Le Tacon (1997) have indeed demonstrated that freezing of *Laccaria bicolor* isolate S238 at −196 or −80 °C did not affect its ability to form mycorrhizas. However, cryopreservation has been reported by several authors to affect the infective potential of a host by fungal pathogens (Zuckerman et al. 1988; Hajek et al. 1995; Mota et al. 2003; Ryan and Ellison 2003; Chetverikova 2009). Therefore, there is a need to demonstrate that the cryopreservation process does not affect the capacity of ECM fungal isolates to associate with a suitable host and to impact their nutrient uptake capacities.

The aim of the present study was, firstly, to evaluate the impact of cryopreservation using the cryovial protocol (Crahay et al. 2013) on the ability of ECM fungal isolates to establish associations with *Pinus sylvestris*. The colonization rates and the concentrations in ergosterol in the roots and in the substrate (external mycelia) of ECM fungal isolates cryopreserved for 6 months were compared to the same isolates stored at 4 °C under axenic culture conditions. Secondly, the uptake of two essential nutrients (inorganic phosphate and ammonium) by mycorrhizal plantlets inoculated with cryopreserved ECM fungal isolates was compared to the uptake capacity of plantlets inoculated with the same fungal isolates preserved at 4 °C under axenic culture conditions and to root uptake of non-mycorrhizal *P. sylvestris* plantlets.

Materials and methods

Fungal material

Eight ECM fungal isolates belonging to six genera and seven species were considered (Table 1). Each isolate was preserved in collection following two methods. In the first method, the isolates were cryopreserved at −130 °C for 6 months following the cryovial protocol (Crahay et al. 2013). Briefly, the mycelium of the ECM fungal isolates was grown in 2-ml sterile cryovials (Sarstedt, Germany) filled with 750 μL of modified Fries medium (MFM) (Colpaert et al. 2000) solidified with 10 gL^{-1} of agar poured in a slope. The cryovials were then incubated at 22–23 °C in the dark for 7 to 9 weeks and subsequently covered by 500 μL of glycerol (10 % *v*/*v*) 1–2 h before cryopreservation. Cryopreservation was then applied using a controlled cooling rate (8 °C min^{-1} from +20 to +4 °C; 1 °C min^{-1} from +4 to −50 °C; 10 °C min^{-1} from −50 to −100 °C), followed by a direct transfer in a deep freezer (SANYO, Japan) at −130 °C for 6 months. For revival, the cryovials with ECM fungal isolates were immersed directly into a water bath at 38 °C for 2 min. Each plug was then carefully transferred on the center of a Petri dish (diameter, 92 mm) on 30 mL of solid MFM and incubated at 22–23 °C for 5 weeks for the slow-growing ECM fungal isolates (i.e., MUCL 52208 and 52210) and 3 weeks for the fast-growing

[1] (MUCL, http://bccm.belspo.be/about/mucl.php)

Table 1 Ectomycorrhizal fungal isolates and number of plantlets (i.e., replicates) considered per isolate in the experiments

ECM fungal isolate	Isolate code[a]	Colonization of the roots and substrate		Uptake of Pi and NH_4^+	
		Cryo[b]	4 °C-stored[c]	Cryo	4 °C-stored
H. crustuliniforme	52208 (Hc1)	9	9	9	9
L. bicolor	52210 (LbicLs1)	9	9	9	9
P. involutus	52215 (Pi2)	9	9	9	9
P. tinctorius	52222 (Pt14)	9	8	9	8
R. luteolus	52199 (E1Rlu)	9	9	8	9
S. bovinus	52173 (P2Sbo)	10	9	2	7
S. luteus	52104 (MG1Slu)	8	9	8	9
S. luteus	52135 (HH18Slu)	8	8	6	6

[a] The MUCL number refers to the code number assigned to the isolate in the Mycothèque de l'Université catholique de Louvain (Louvain-la-Neuve, Belgium) and the number between brackets refers to the original code of the isolate. The isolates were collected by Prof. J. Colpaert in the province of Limburg (Belgium) from *Pinus* sp. except *P. tinctorius* MUCL 52222 for which the collector, locality, and host plant are unknown. For more details see Crahay et al. (2013)

[b] Number of plantlets inoculated with ECM fungal cultures preserved by cryopreservation at −130 °C for 6 months

[c] Number of plantlets inoculated with ECM fungal cultures preserved on nutrient agar Petri dishes stored at 4 °C

isolates (i.e., MUCL 52215, 52222, 52199, 52173, 52104, and 52135). Five viable cryopreserved cultures that covered approximately half of the Petri dishes were then selected for the phenotypic stability assessment.

In the second method, the ECM fungal isolates were preserved on nutrient agar Petri dishes stored at 4 °C. After 5 months, the fungal isolates were transferred onto fresh solid MFM at pH4.8 and incubated for 5 or 3 weeks at 22–23 °C as above.

Plant material

P. sylvestris seeds were washed with Tween 20 and surface-disinfected with 10 % H_2O_2, sown in expanded perlite (Sibli S.A., Andenne, Belgium), and watered with Ingestad's balanced nutrient solution for *P. sylvestris* (Ingestad and Kähr 1985). The weight proportions of the macronutrients in the solution were 100 N/65 K/15 P/6 Ca/6 Mg/9 S (see Nylund and Wallender (1989) for the exact formulae of the stock solutions). The plantlets were maintained in a growth chamber at 22/18 °C (day/night), 65 % relative humidity with a photoperiod of 12 $h\,day^{-1}$, and under a photosynthetic photon flux of 300 $\mu mol\,m^{-2}\,s^{-1}$.

Inoculation of *P. sylvestris* plantlets

A sandwich technique was used to inoculate 5-week-old *P. sylvestris* seedlings with actively growing mycelia from the ECM fungal isolates preserved by cryopreservation or stored at 4 °C under axenic conditions as described by Adriaensen et al. (2005). Five Petri dishes per isolate and per method of preservation (by cryopreservation or storage at 4 °C) were filled with MFM (approximately 30 mL) solidified with 10 $g\,L^{-1}$ of agar covered with sterile cellophane sheets (Hutchinson, France). In order to obtain a uniform mycelium coverage of the cellophane sheet, seven plugs were inoculated per Petri dish for the slow-growing ECM fungal isolates and incubated at 23 °C for about 3 weeks, while two to three plugs were inoculated for the fast-growing ECM fungal isolates and incubated for 1–2 weeks. Eight to ten plantlets per isolate and per method of preservation were inoculated depending on the mycelium growth onto the cellophane sheet (see Table 1 for details). Nine non-mycorrhizal control plantlets were treated similarly, but without cellophane sheet covered with mycelium.

After 3 to 4 days of incubation, the plantlets were transferred in 140-mL containers filled with perlite and incubated as described above. In addition, five containers were filled with perlite but without plantlets (control). Plantlets were watered three times a week with Ingestad's nutrient solution.

Nutrient uptake capacity

At 8 to 9 weeks after inoculation, the short-term uptake of inorganic phosphate (Pi) and ammonium (NH_4^+) by the plant–fungus associates was determined from the depletion of those nutrients in a liquid nutrient solution circulating through the plant containers (Colpaert et al. 1999; Van Tichelen et al. 1999). After 15 min, 1, 2, 3, and 4 h, Pi and NH_4^+ concentrations were assessed by colorimetric analysis using a flow injection analyzer (Lachat Instruments, USA, QuickChem® Methods 10-115-01-1-A and 10-107-06-1-C). From the nutrient depletion curves, uptake rates were calculated after 3 h of nutrient solution circulation.

Harvest and analyses

The 16-week-old plantlets were harvested. A sample of roots from each plantlet was randomly collected and scanned (scanner HP Scanjet 3970, USA) to evaluate the ECM root colonization. One hundred fine roots were randomly examined using the image analysis software IMAGEJ 1.45 s (National Institute of Health, USA), and the number of tips colonized by the ECM fungal isolates were counted.

Samples of roots and perlite from each plantlet were frozen in liquid nitrogen and subsequently freeze-dried for determination of ergosterol content. Ergosterol is a fungal-specific biochemical marker of metabolically active mycelial biomass which has been widely used to estimate the colonization of soil and plant tissues by ECM fungi (Ekblad et al. 1998; Nylund and Wallander 1992). Ergosterol was extracted from freeze-dried roots and perlite subsamples and quantified by high-performance liquid chromatography as described by Nylund and Wallander (1992).

Statistical analysis

In order to test whether the method of preservation had an effect on the colonization level, ergosterol concentrations, and the Pi and NH_4^+ uptake rates, the data were analyzed with a mixed-model ANOVA. Colonization percentages were subjected to arcsine square root transformation, while uptake rates were subjected to a log transformation prior to analyses. The method of preservation was analyzed as fixed effects, and the ECM fungal isolates were used as a random effect. When interaction between the method of preservation and the fungal isolates was detected, the interaction was included as a random effect in the mixed model. Each isolate was then considered separately, and the effect of the method of preservation was determined by Welch's ANOVA, assuming unequal variance between samples. For all tests, P values <0.05 were considered as significant. The analyses were performed with the Statistical Analysis System software (SAS enterprise Guide 4.2).

Results

ECM fungal root colonization and ergosterol concentration in roots and perlite

The colonization rate and ergosterol concentration in the root systems of *P. sylvestris* associated with ECM fungal isolates are presented in Fig. 1a, b, respectively. Overall, the method of preservation had no significant effect on ECM fungal root colonization (P value=0.6945) and ergosterol concentration in roots (P value=0.3064). However, for some ECM fungal isolates, significant differences were noticed in the colonization ability between cryopreserved and 4 °C-stored cultures. The plantlets inoculated with cryopreserved cultures of *L. bicolor* MUCL 52210 exhibited a significantly higher colonization rate than those inoculated with the 4 °C-stored cultures (P value=0.0450), whereas the reverse was noticed with *Suillus bovinus* MUCL 52173 (P value=0.0061) (Fig. 1a). Identically, the ergosterol concentration in roots was significantly higher in the plantlets inoculated with cryopreserved cultures of *Hebeloma crustuliniforme* MUCL 52208 (P value=0.0059), *Paxillus involutus* MUCL 52215 (P value=0.0008), and *Pisolithus tinctorius* MUCL 52222 (P value=0.0302) as compared to the plantlets inoculated with the 4 °C-stored isolates (Fig. 1b). On the contrary, the plantlets inoculated with cultures of *S. bovinus* MUCL 52173 cryopreserved for 6 months exhibited significant lower root ergosterol concentrations than those inoculated with the 4 °C-stored cultures (P values=0.0097).

There was no significant difference in the concentration of ergosterol in the substrate between the plantlets colonized by the ECM fungal isolates cryopreserved or preserved at 4 °C (P value=0.3103). However, with *S. bovinus* MUCL 52173, the ergosterol concentration in perlite was significantly lower for the cultures preserved by cryopreservation even though it could not be shown statistically because all concentrations in perlite were null for the cryopreserved samples (Fig. 1c). No differences were noticed for the seven other isolates.

In the non-mycorrhizal plantlets, no root colonization was detected, and the ergosterol concentrations in the roots as well as substrate were null. No ergosterol was detected in the substrate of the five controls.

Nutrient uptake capacities

For some isolates, we observed that a number of replicates failed to colonize the root system. These non-colonized replicates were discarded from the analysis on nutrient absorption to adequately evaluate the effect of the mode of preservation on this physiological property (Table 1).

Overall, the Pi depletion curves in the circulating solutions of the plantlets inoculated with the cryopreserved ECM fungi or inoculated with the 4 °C-stored ECM fungal isolates were similar, while it was less important in the non-mycorrhizal plantlets as compared to the mycorrhizal plantlets. The Pi concentrations in the solution circulating through the controls (i.e., containers without plants) remained unchanged during the tests. Consequently, the Pi uptake rates, estimated after 3 h of circulation, did not differ significantly between the plantlets inoculated with the cryopreserved ECM fungi and the plantlets inoculated with the 4 °C-stored ECM fungal isolates (P value=0.7011) but were significantly higher than those of the non-mycorrhizal plantlets (P values<0.0001 and <0.0001 for plantlets colonized respectively with the cryopreserved and 4 °C-stored ECM fungal isolates).

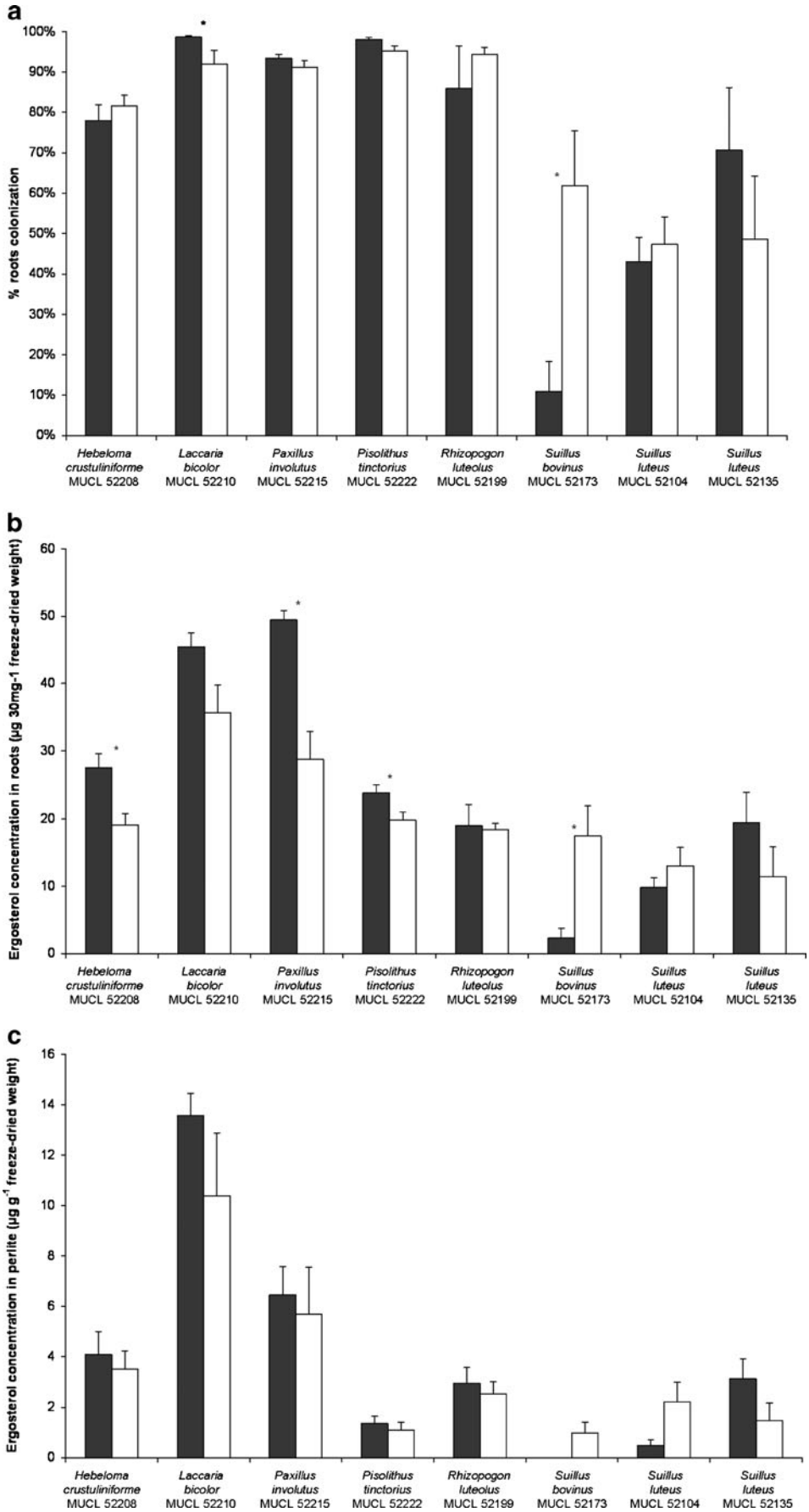

Fig. 1 Colonization rates (**a**), ergosterol concentration in roots (**b**), and perlite (**c**) of *P. sylvestris* plantlets inoculated with ECM fungal isolates preserved by cryopreservation at −130 °C for 6 months (*black histograms*) or on nutrient agar Petri dishes and stored at 4 °C (*white histograms*). *Error bars* represent the standard error of the mean. An *asterisk* represents significant differences at $P<0.05$ (Welch's ANOVA) between cryopreserved cultures and non-cryopreserved cultures

When the effect of the mode of preservation on the Pi uptake by mycorrhizal plantlets was examined for each isolate separately, significant differences were only observed with the plantlets inoculated with *S. bovinus* MUCL 52173 (Fig. 2a). For this isolate, a significant higher Pi uptake rate was observed for the plantlets associated with the 4 °C-stored cultures as compared to the cryopreserved cultures (P value=0.0014). Most ECM fungal isolates cryopreserved or stored at 4 °C exhibited significantly higher Pi uptakes rates than the non-mycorrhizal control, except the plantlets inoculated with cryopreserved and 4 °C-stored cultures of *Suillus luteus* MUCL 52104 and with 4 °C-stored cultures of *S. luteus* MUCL 52135 that did not exhibit significantly different rates.

Overall, the NH_4^+ depletion curves were similar for the plantlets inoculated with the cryopreserved ECM fungi and those inoculated with the 4 °C-stored ECM fungi, while the depletion of NH_4^+ in the circulating solution was less important in the non-mycorrhizal plantlets compared to the mycorrhizal plantlets. The NH_4^+ concentration in the solution circulating through the controls (i.e., containers without plants) remained unchanged throughout the experiment. Consequently, the NH_4^+ uptake rates, estimated after 3 h of circulation, did not differ significantly between the plantlets inoculated with the cryopreserved ECM fungal isolates and the plantlets inoculated with the 4 °C-stored ECM fungal isolates (P value=0.7936) but were significantly higher than that of the non-mycorrhizal plantlets (P values<0.0001 and<0.0001 for plantlets colonized respectively with the cryopreserved and 4 °C-stored ECM fungal isolates).

When the effect of the mode of preservation on the NH_4^+ uptake by mycorrhizal plantlets was examined for each isolate separately, significant differences were only observed with the plantlets inoculated with *S. luteus* MUCL 52104 (Fig. 2b). For this isolate, a significant higher NH_4^+ uptake rate was observed for plantlets associated with 4 °C-stored cultures of *S. luteus* MUCL 52104 as compared to the cryopreserved cultures (P value=0.0085).

Most isolates cryopreserved or stored at 4 °C exhibited significantly higher NH_4^+ uptake rates than the non-mycorrhizal control, with the exception of four associations, i.e., the plantlets inoculated with cryopreserved and 4 °C-stored cultures of *S. bovinus* MUCL 52173, with cryopreserved cultures of *S. luteus* MUCL 52104, and with cultures of *S. luteus* MUCL 52135 preserved at 4 °C.

Discussion

Cryopreservation at ultralow temperature is nowadays considered as the most suitable method to maintain phenotypic and genetic stability of a large amount of filamentous fungi (Smith 1998) including ECM fungi (Crahay et al. 2013). However, the impact of cryopreservation on important phenotypic characteristics (e.g., root colonization and nutrient transport) of ECM fungi is poorly documented. So far, most of the cryopreservation studies have been conducted on axenic cultures of ECM fungi, while the ECM fungi in their symbiotic phase have seldom been considered. Here, we investigated the ability of eight ECM fungal isolates cryopreserved at −130 °C for 6 months or preserved at 4 °C on synthetic medium to establish symbiosis with *P. sylvestris* and to take up Pi and NH_4^+ from the plant growth circulating solution.

For most ECM fungal isolates tested, the mode of preservation had no significant effect on the ability to colonize the root system of the *P. sylvestris* seedlings. These observations are consistent with the study of Corbery and Le Tacon (1997). These authors have shown that cryopreservation of the ECM fungal isolate *L. bicolor* S238 at −196 or −80 °C did not affect its ability to form mycorrhizas. Interestingly, Lalaymia et al. (2012) reported that arbuscular mycorrhizal fungi, another important group of mycorrhizal fungi, remained viable after 6 months of cryopreservation at −130 °C and were able to reproduce the fungal life cycle in vitro. Similarly, mycorrhizal colonization mostly enhanced Pi and NH_4^+ uptake in pine seedlings, and this increase was not affected by the mode of preservation for most of the ECM fungal isolates tested. Cryopreservation therefore appeared as a reliable method for the preservation of important phenotypical characteristics (i.e., root colonization and nutrient acquisition) of most ECM isolates.

For four ECM fungal isolates (i.e., *H. crustuliniforme* MUCL 52208, *L. bicolor* MUCL 52210, *P. involutus* MUCL 52215, and *P. tinctorius* MUCL 52222), cryopreservation appeared more adequate than storage at 4 °C in terms of maintenance of ECM-forming ability already after 6 months of storage. Indeed, the isolates preserved by storage at 4 °C exhibited lower ergosterol concentrations in the roots or a reduced colonization rate when associated with pine roots as compared to the same isolates cryopreserved for 6 months. It can be hypothesized that the higher reliability of cryopreservation compared to the preservation on nutrient agar Petri dishes stored at 4 °C would have even been more marked after a longer period of storage and repeated subculturing. Indeed, Laiho (1970) and Marx and Daniel (1976) observed that ECM fungal isolates lost their ability to colonize the roots after several years of continuous subcultures even if Thomson et al. (1993) reported loss after only few months in axenic cultures.

Conversely, few isolates were impaired by the cryopreservation process. Only *S. bovinus* MUCL 52173 failed to colonize the roots and exhibited a reduced capacity to take up Pi following cryopreservation, while *S. luteus* MUCL 52104 exhibited a decreased ability to take up NH_4^+. This suggested that cryopreservation is a complex phenomenon that can variably affect ECM fungi.

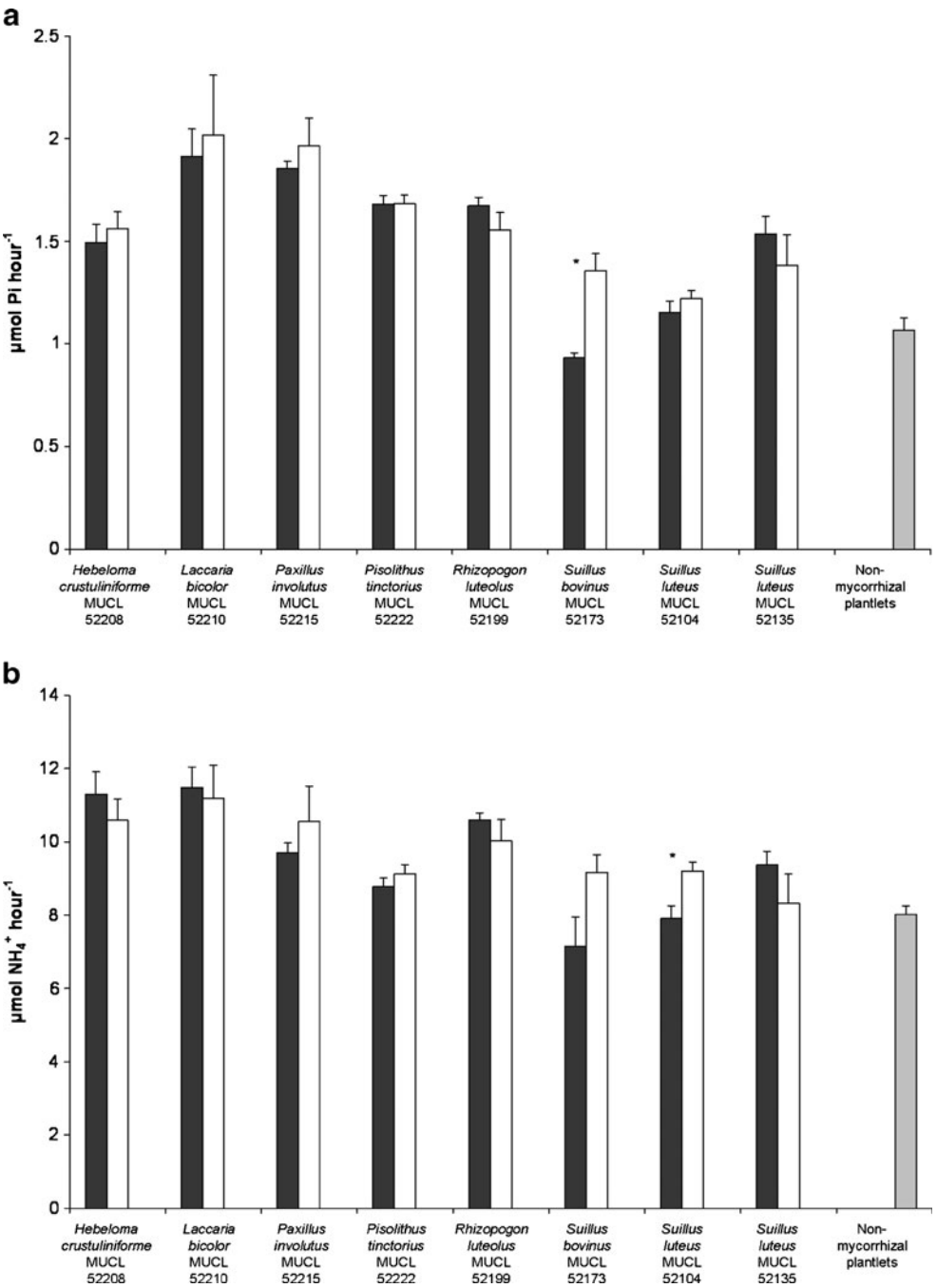

Fig. 2 Pi (**a**) and NH_4^+ (**b**) uptake rates in *P. sylvestris* plantlets inoculated with ECM fungal isolates preserved by cryopreservation at −130 °C for 6 months (*black histograms*) or on nutrient agar Petri dishes stored at 4 °C (*white histograms*) or in non-mycorrhizal *P. sylvestris* seedlings (*gray histograms*). *Error bars* represent the standard error of the mean. An *asterisk* represents significant differences at $P<0.05$ (Welch's ANOVA) between cryopreserved cultures and non-cryopreserved cultures

Surprisingly, in our experiment, the loss of ability of *S. bovinus* MUCL 52173 to form mycorrhizas did not affect all cryopreserved cultures of this isolate since two plantlets exhibited fine roots and high ergosterol concentrations although cryopreservation was conducted under the same conditions (freezing rate, thawing and revival regime,…) and at the same time for all cultures of this isolate. Similarly, the loss of ability of this isolate to form mycorrhizas after storage at 4 °C did not affect all the subcultures despite that all cultures used for inoculation of the *P. sylvestris* seedlings originated from the margin of the same ECM fungal culture. This indicated that there are differences in the colonization ability between different subcultures originating from the mycelium of a single ECM culture. Because in our experiment, cryopreserved cultures have undergone different subculturing steps before and after cryopreservation (i.e., pre-cultures onto Petri dishes, inoculation of the pre-cultures in the cryovials, and transfer of the cryopreserved cultures on agar Petri dishes for revival), a loss of ECM-forming ability could have affected some subcultures of the *S. bovinus* isolate at each transfer of culture. Consequently, the culturing steps required by the

cryopreservation process might be partially responsible for the loss of colonization potential observed for cryopreserved cultures of *S. bovinus* MUCL 52173. The fact that phenotypic and genetic variations observed in plants following cryopreservation are mostly attributed to the pre- and post-culture procedures rather than the cryopreservation process itself supports this hypothesis (Harding 2010).

Finally, the response to cryopreservation in terms of maintenance of phenotypic integrity appears to differ not only between the ECM fungal species but also between isolates of a single species. For instance, the uptake of NH_4^+ by *S. luteus* MUCL 52135 was unaffected by the cryopreservation process, whereas a significant decrease was observed for the isolate MUCL 52104. This intraspecies difference was also reported by Ryan et al. (2001) who observed that for the entomopathogenic fungus *Metarhizium anisopliae*, cryopreservation and lyophilisation differentially affected the secondary metabolite profiles of different isolates.

In conclusion, the cryovial protocol appeared as a promising suitable cryopreservation method not only to reach high viability rates as demonstrated in Crahay et al. (2013) but also to maintain important phenotypic characteristics such as the ECM-forming ability and the capacity to take up Pi and NH_4^+. To confirm the reliability of the cryovial protocol, the evaluation of genotypic stability of ECM fungal cultures following cryopreservation as well as longer periods of storage need to be tested. In order to evaluate genetic stability, amplified fragment length polymorphism (AFLP) fingerprint patterns generated for ECM fungal isolates preserved by cryopreservation should be compared to the same ECM fungal isolates preserved on nutrient agar Petri dishes stored at 4 °C.

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